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Bioprospection And By-Product Utilization Of Juniperus Virginiana

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BIOPROSPECTION AND BY-PRODUCT UTILIZATION OF *JUNIPERUS VIRGINIANA*

By

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Experiments were conducted to study variation in the amount of podophyllotoxin in *J. virginiana* across nine physiographic regions of Mississippi. The aim was bioprospection of podophyllotoxin from *J. virginiana* across Mississippi. Experiments were also conducted to utilize biproducts (i.e. needles) as a source of podophyllotoxin and essential oil. Dual extraction of both from the same plant material was obtained in order to develop economic protocol for industrial utilization. Three different experiments were conducted for the bioprospection and byproduct utilization of *J.virginana.*

Experiment I: Study of variation in the amount of podophyllotoxin across nine physiographic regions and the study of contributing factors for the variability.

Experiment II: Explore different plant parts as source of podophyllotoxin

Experiment III: Establish a dual extraction protocol for extraction of podophyllotoxin and essential oil from *J. virginiana.*

DEDICATION

To my parents

ACKNOWLEDGMENTS

I wish to thank my major Professor Dr. Valtcho Jeliazkov, for allowing me to take this project from an idea conceived to an idea established. This journey from objectives to conclusions has indeed been a rewarding experience because of his nature to welcome new ideas, the experimental independence he offered me to develop procedures and his experienced suggestions at every point to exclude short-comings in the project.

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Without the technical help of Mr. Thomas Horgan, Ms. Marie Rogers, Ms. Amber Callahan Reichley and Mr. Solomon Green III, I would have gone juggles over putting things together. I thank them for all the help.

Without the technical help of Mr. Thomas Horgan, Ms. Marie Rogers, Ms. Amber Callahan Reichley and Mr. Solomon Green III, I would have gone juggles over putting things together. I thank them for all the help.

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CHAPTER 1.

INTRODUCTION

Juniperus virginiana L. is an evergreen tree that grows in abundance all over United States and in parts of Canada. It is used in timber industry and for the production of essential oil from heartwood. *J. virginiana* is a good source of two important products, podophyllotoxin (an anti-cancer compound) and essential oil. The utilization of the tree for obtaining these two important products was studied in the present project. The objectives set upfront follow.

1.1. Objectives and Approach of the present project

1.1.1 Bioprospection for podophyllotoxin in *J. virginiana*

Mississippi shows variation in physiographic regions ranging from rich alluvial Delta, the calcium rich soils of Blackland Prairie, hilly regions of Tishomingo Hills to the plains of North Central plateau. With such diversity in physiography, soil type, elevation and climatic factors, variability in the amount of active constituents may not be impossible. The present project aimed to study the chemo-variation with respect to the content of podophyllotoxin in a medicinally and aromatically important plant, *J. virginiana*, sampled across nine physiographic regions of the state of Mississippi.

Considering the fact that there will be significant variation in the environmental factors across these regions, the study of these factors with regard to the amount of active constituents was also thought to be an integral part of the present project. The factors under study were soil type, soil physical properties, soil nutrients and associate species. The project thus aimed to study the correlation of all these with regard to the biosynthesis of the active compound, podophyllotoxin. The study will enable the use of this data for selecting the highest yielding raw material and physiographic region, thus ensuring industrial quality. Also it may help to establish the high yielding accession and in developing cultivation practices for the maximum utilization of *J. virginiana*.

1.1.2 *J. virginiana* and waste product utilization

Currently, heartwood oil has been studied extensively. However, not much has been researched about utilizing other plant parts like bark, sapwood or leaves, all of which are waste of the timber industry, which only uses the wood. The study of these plant parts as a source of essential oil and podophyllotoxin as a means of by-product utilization is also a part of the current project**.**

1.1.3 Simultaneous extraction of podophyllotoxin and essential oil

Leaves are a known source of both podophyllotoxin and essential oil. Extraction of both these products simultaneously, is also an important objective of the present research on *J. virginiana.*

CHAPTER 2.

LITERATURE REVIEW

2.1.Introduction of *J. virginiana*

2.1.1. Botany and Classification

The genus Juniperus belongs to the Cupressaceae family of plant taxa. There are 28 species and 41 accepted taxa under the genus Juniperus. *J. virginiana* is one amongst this genus of Juniperus.

J. virginiana L., (Figure 1) is a medium-sized evergreen tree. It is dioecious or rarely monoecious tree with a height of 10-20 m tall (McGregor *et al,* 1986; Stephens, 1973). The evergreen tree is shaped like a pyramid or column, with reddish-brown to grayish colored fibrous bark. Leaves are opposite, simple, green or blue-green, scale-like, closely appressed and overlapping in one direction. The leaves, also referred to as needles, are 0.2-0.3 cm broad and 0.6-1.2 cm long. Male and female trees are separate with male and female cones on separate trees (Figure 2). The male cones are staminate, yellowish-brown, papery, solitary at the tips of branchlets, ovoid to ellipsoid, and 0.2-0.4 cm long. The female cones are solitary at the tips of branchlets, dark blue or bluish-purple, waxy and berrylike. They are 0.4-0.7 cm long. The female cones ripen from September through October and look like berries which are about 4-6 mm in diameter.

There are 1-3 seeds per cone. The seeds are yellow-brown and round, 2-4 mm in diameter, ridged near the base, and sometimes shallowly pitted.

2.1.2. Distribution and occurrence

J. virginiana is native to Eastern North America, where it occurs frequently on limestone derived soils and is cultivated in Wyoming and Colorado for shelterbelts and ornamental uses below 6,000 feet of altitude. This species has a wide distribution and is found on many types of soil ranging from acid sands to those derived from limestone. It grows best on dry soils in full sunlight, and is winter hardy and tolerant to droughty and salty soils. Like most junipers, it is very slow growing and is moderately long lived (USDA factsheet, 2002).

J. virginiana is distributed throughout United States of America and many provinces of Canada. It has been reported in Alaska, Arizona, Colorado, Connecticut, Washington DC, Delaware, Florida, Georgia, Iowa, Illinois, Indiana, Kansas, Kentucky, Louisiana, Massachusetts, Maine, Michigan, Minnesota, Montana, Mississippi, North Carolina, North Dakota, Nebraska, New Hampshire, New Jersey, New York, Ohio, Oklahoma, Oregon, Pennsylvania, Rhode Island, South Carolina, South Dakota, Texas, Tennessee, Virginia, Vermont, Wisconsin and West Virginia (Figure 3).

There are various ornamental cultivars developed that are available through most nurseries. Cultivars include: Baker's Blue, Blue Mountain, Brodie, Burkii, Canaerti, Cupressifolia, Dundee, Emerald Sentinel, Glauca, Gray Owl, Hillspire, Idyllwild, Manhattan Blue, Mission Spire, Nova, Pendula, Patt River, Princeton Sentry, Royo, Silver Spreader, Stover, and Taylor (USDA factsheet, 2002).

2.1.3. Economic Importance

J. virginiana is planted in the outer rows of multi-row plantings, where it will not be over topped by taller trees, to serve as a good windbreak. It can be used in single-row windbreaks when a dense, medium height barrier is desired. *J. virginiana* is often used as ornamentals for their evergreen foliage. Its year-long coloration and attractiveness to wildlife adds a good variety to recreational plantings. Most cemetery plantings include old trees and many younger dwarf junipers. All of the native junipers are valuable ornamental species, and many horticultural varieties have been developed. The fruits and young branches contain aromatic oil that is used in medicines (USDA plant fact sheet, 2002). The wood is well known for its close-grained, aromatic, and durable wood, which is used for furniture, interior paneling, novelties, and fence posts.

J. virginiana provides food and cover for numerous birds and mammals such as pheasant, mule deer, hoofed browsers and whitetail deer are very common during winter. Birds like cedar wax wing feeds on the bluish-purple berry-like fruits. Other birds that are found habitants of junipers are chipping sparrows, robins, song sparrows, and mockingbirds, juncos, myrtle warblers and sparrows of various kinds.

J. virginiana has been used by many tribes for incense in purification and ritual (Kindscher, 1992), as for numerous tribes, the tree symbolizes the tree of life and is hence burned in sweat lodges and purification rituals. The tribes like Cheyenne, Flathead, Nez Perce, Kutenai, and Sioux made a tea from branches and fleshy cones to treat colds, fevers, tonsillitis, and pneumonia and as a sedative to a hyperactive person (Hart, 1976). The Blackfeet tribe made a tea from the berries to stop vomiting (Kindscher, 1992). The leaves were also rubbed on affected parts for arthritis and rheumatism. They also made

root tea as a general tonic and for stiff backs or backache (McClintock, 1909; Johnston, 1970; Hellson, 1974). Women of the Cheyenne tribe drank the tea to speed up delivery during childbirth (Grinnell, 1962). As a cure for asthma, the Gros Ventres tribe ate whole berries or pulverized them and boiled them to make a tea. They also made a preparation from the leaves mixed with the root, which they applied topically to control bleeding (Kroeber, 1908). The Crow tribes drank this medicinal tea to check diarrhea and to stop lung or nasal hemorrhage. Crow women drank it after childbirth for cleansing and healing (Hart, 1976).

The wood of *J. virginiana* is very durable, and has been used for lance shafts and bows, flutes etc. The bark mats were used for roofing temporary structures, for partitions, floor mats and wrappings, and for various purposes in the canoes (USDA factsheet, 2002).

J. virginiana apart from many of its uses is primarily well-known for its use of durable, termite and insect resistant (redwood). The heartwood (Figure 4) is also being used for commercial production of essential oil, commonly termed as cedarwood oil. The distilled oil of *J. virginiana* has been officially listed as a reagent in the *U.S. Pharmacopoeia* since 1916. It has been extensively studied since then for its oil composition and yield (Coleman and Lawrence, 1997; Payne *et al,* 1999; Eller and King, 2000; Dunford *et al*, 2006). U. S. Pharmacopoeia, from 1820 to 1894, listed for the first time the young leafy twigs of *J. virginiana* as a diuretic (Kindscher, 1992). Tumor inhibitor podophyllotoxin was identified for the first time in *J. virginiana* by Kupchan *et al* in 1965. Later *J. virginiana* was studied as an alternate source for podophyllotoxin to that of *Podophyllum* spps (Bedir *et al*, 2002; Canel *et al,* 2000).

Figure 1 *J. virginiana* habitat

Figure 2 Female and male plants of *J. virginiana*

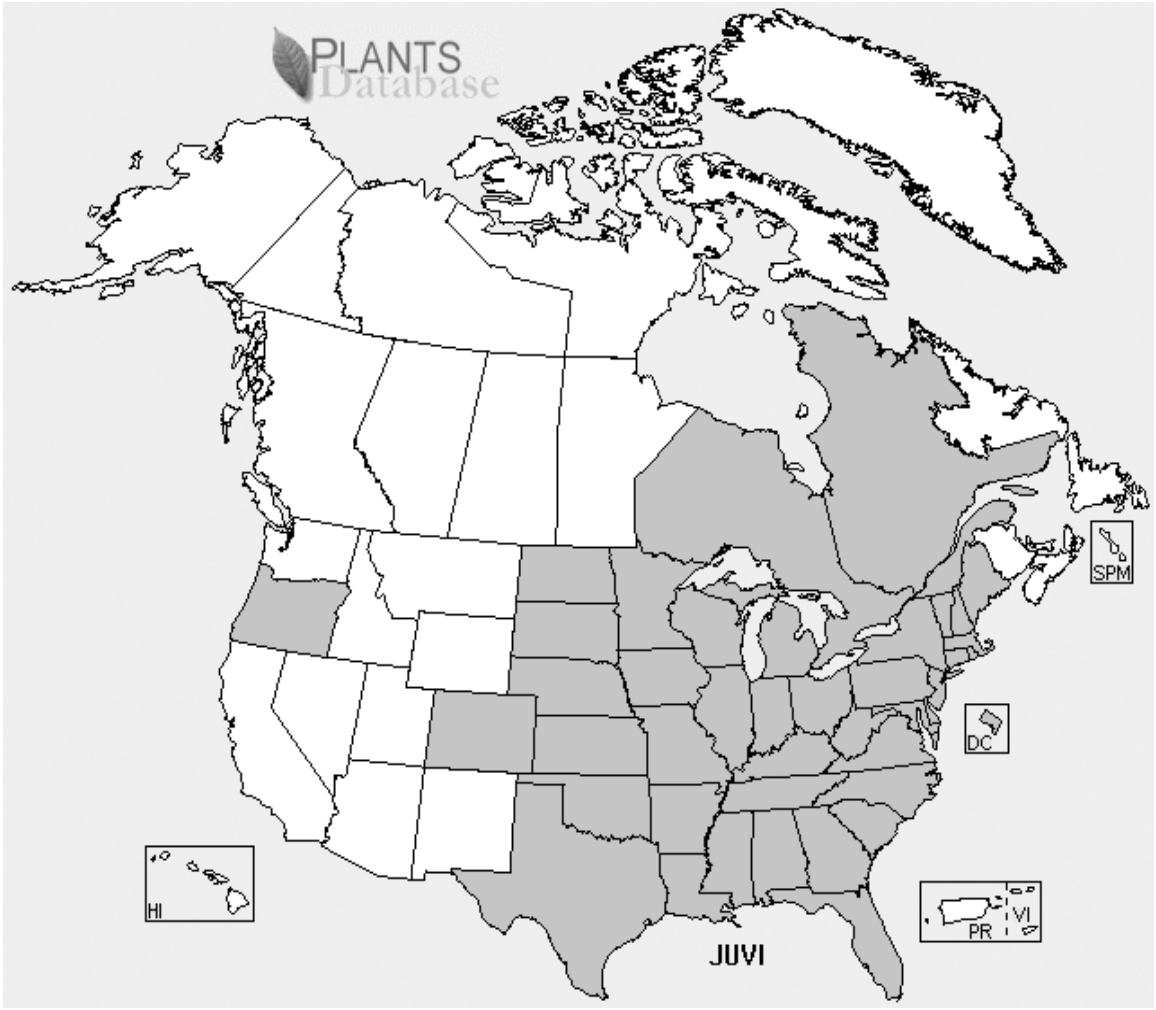


Figure 3 Distribution of *J. virginiana* in United States of America

Courtesy: USDA-NRCS http://plants.usda.gov/java/profile?symbol=JUVI

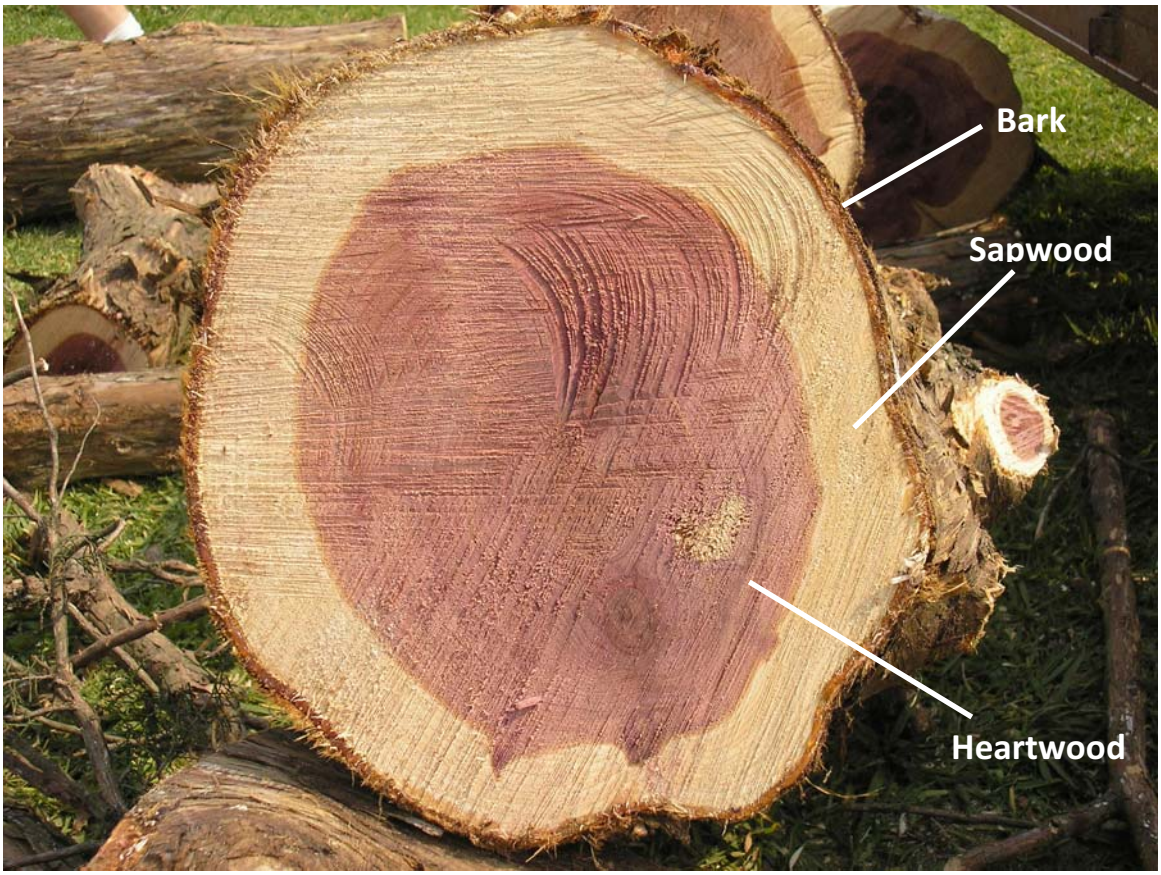


Figure 4 Cross section of stem of *J. virginiana,* showing bark, sapwood and heartwood

2.2 Bioprospection

2.2.1. Definition and application

There is an enormous degree of intra-specific variation observed in plants at morphological, genetic and chemical/physiological level (Meloni *et al*, 2006; Muzquiz *et al*, 1994; Purohit *et al*, 1999; Roger *et al*, 2008; Wheeler *et al*, 2007). These variations are a result of genetic make-up and environmental factors, either one or both. The factors of environmental variability are geographic variations which consist of a combination of climatic factors, soil elements and soil type. All these factors together vary the amount of

active constituents and overall composition of plant products like essential oils. The physiography and topography varies with location; also topographical features, temperature, rainfall and soil elements in the given location, cumulatively affect the synthesis of active constituents. As a result, plants acquired from different locations have a high tendency to show variations. Other factors like associate species, sex of the plant, age of the plant, season of harvest, light availability etc. are also shown to affect the concentration of active constituents. For example, variation in the concentration of camptothecin (CPT) was observed in relation to leaf, branch, tree age, season, and leaf drying method in *Camptotheca acuminata* Decaisne saplings (Liu *et al*, 1998). Seasonal variation has been observed in the content of artemisinin in plants of *Artemisia annua* collected from different geographical origins, proving the existence of chemotypes (Stewart, 2003). Variation in essential oil composition was observed in *A. annua* in different cultivars cultivated in Finland (Holm *et al*, 1997). Forty nine genotypes of *Lupinus alba*, collected from different locations and countries, varied in alkaloid composition (Muzquiz *et al*, 1994). Forty two Neem ecotypes of India have shown a wide variation in the content of oil, and their physicochemical characteristics (Kumar and Parmar, 1996). In tobacco, the polyphenol concentrations were observed to significantly vary with location, stalk position and season in a cultivar study of tobacco (Williamson and Gwynn, 1982). Differences in podophyllotoxin concentration were observed in different populations of *Podophyllum hexandrum* (Purohit *et al*, 1999) and also different accessions of *Podophyllum peltatum* (American mayapple) collected from different locations (Bedir *et al*, 2002).

Study of variations is important in order to standardize an industrial process. Such variations largely affect the drug potential of a particular variety/accession, thus leading to variability in the quality of the plant material/raw material for extraction of valuable chemicals with drug/flavor/fragrance potential. Standard methods of isolation of active constituents hence may lead to variable yields of the pure compound due to variation in the source plant material. For instance, the level of polyphenols in tobacco affects the composition of smoke, thus affecting the quality of tobacco (Wallart *et al*, 2000). The amount of the active constituent also affects the bioactivity of species and variations of chemical profile within a species could lead to variability in bioactivity. For example, study of insect growth inhibition of *Spodoptera litura* revealed a wide variation in the EC_{50} of the oils of different accessions of *Azadiracta indica* (Kumar and Parmar, 1996).

Identification and mapping of chemo-variations across different physiographic regions, study of factors affecting this variability and pooling of all results of suitability for the highest production of the desired compound, collectively can be referred to as bioprospection. Identification of chemo-variability helps to predict the amount of active constituents in a particular accession obtained for a particular physiographic region. In addition it also helps identify the most suitable environmental factors for the production of these active constituents. These data can further be used for conservation, cultivation and further utilization of the species.

2.2.2. Bioprospection for podophyllotoxin

Podophyllotoxin (Figure 5) is a natural lignan that is being used as a precursor to semi-synthetic anti-cancer drugs etoposide and teniposide (Figure 5b and 5c). These

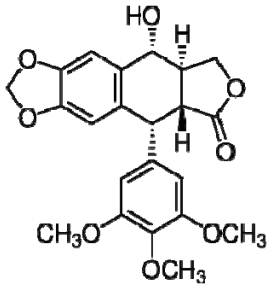
compounds are the aldehyde condensation products of 4`-demethylpipodophyllotoxin glucoside (DEPG). These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemia (Stahelin and Wartburg, 1991; Imbert, 1998). In addition podophyllotoxin is also the precursor to a new derivative of CPH 82 tested for rheumatoid arthritis in Europe and other derivatives for the treatment of psorosis and malaria (Leander and Rosen, 1988; Lerndal and Svensson, 2000). Podophyllotoxin is also used to treat genital warts (Beutner, 1996) and as an immune-stimulatory agent (Pugh *et al*, 2001).

Podophyllotoxin is primarily obtained from the rhizomes and roots of *Podophyllum emodi* syn. *P. hexandrum* (Indian mayapple), considered endangered due to extensive harvest from the wild (Foster, 1993). The leaf blades of *Podophyllum peltatum* (American mayapple) and *J. virginiana* were studied and discovered as alternative sources of podophyllotoxin (Cushman *et al*, 2001). Other genera like *Linum, J. , Hyptis, Teucrium, Nepeta, Dysosma, Jeffersonia, Thymus and Thuja* are also known to produce podophyllotoxin (Kupchan *et al,* 1965; San Feliciano *et al*, 1989,a,b; Broomhead and Dewick, 1990 a,b; Yu *et al*, 1991; Kuhnt *et al,* 1994; Konuklugil, 1996 a,b; Muranaka *et al,*1998).

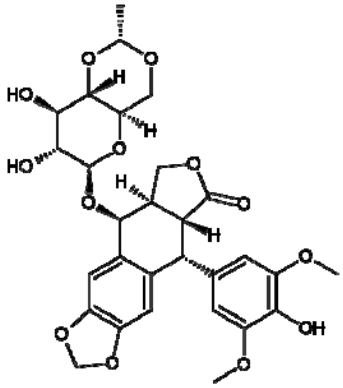
Bedir *et al* (2002) observed that the content of podophyllotoxin in mayapple leaves and *J. virginiana* needles show 21%:3% ratio of content of podophyllotoxin for mayapple:redcedar. A bioprospection study showed that the content of podophyllotoxin varied significantly among genotypes of American mayapple harvested from the wild, ranging from 1.1 to 56 mg/g on a dry weight basis (Moraes *et al*, 2002).

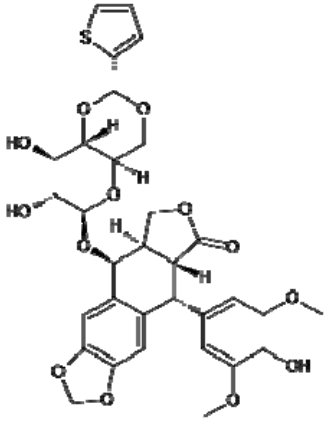
2.2.3. Study of variation in *J. virginiana*

Earlier studies on the variation in *J. virginiana* show clinical variation based on terpenoid data in different populations collected along the transect from northeast Texas to Washington DC (Flake *et al*, 2007). Significant difference was observed in the composition of leaf essential oil obtained from Texas and Ontario accessions (Semen and Hiziroglou, 2005). Also variation in essential oil composition was studied with respect to age of the tree (Dunford *et al*, 2006). Significant variation in leaf oil of *J. virginiana* was found between sexes and between habitats within a single geographic region (Setzer *et al*, 1992).



Podophyllotoxin





Etoposide Teniposide

Figure 5 Structure of podophyllotoxin, etoposide and teniposide

2.3 Physiographic Regions of Mississippi

Topography and soil largely influence the natural vegetation in an area. Factors like rainfall, temperature, humidity and soil composition play an important role in the plant growth and synthesis. The amount of water held in the soil, the types and quantity of soil nutrients, slope and aspect of an area, day/night temperature fluctuations and atmospheric humidity, all of these affect the synthesis and growth of plants. The topography of Mississippi is quite diverse, ranging from only a few feet above sea level along the Gulf of Mexico to elevation of 805 feet at the Tishomingo Hills. Considering

this diversity of physiography, the study of chemo-variation (if any) across these regions seemed an essential part of research in the utilization of *J. virginiana*. The nine physiographic regions of Mississippi that were considered for study in the present study are Piney Hills, Jackson Prairies, North Central Plateau, Flatwoods, Pontotoc Ridge/Pontotoc Hills, Black Prairie/Blackland Prairie, Tishomingo Hills/Fall Line Hills and Bluff/Loess Hills (Figure 6).

1. **Pine Hills/Piney Hills:** This region is flat to hilly. It is characterized by marine deposits of sandy, loamy and clayey materials that often contain gravel. Depending on the vegetation the region may be categorized dry or wet regions.
2. **Jackson Prairies:** The Jackson Prairies are distinguished by rolling to hilly topography with soils consisting from sand, loam or clay. Small prairie remnants, with typical prairie flora are scattered throughout the area.
3. **North Central Plateau:** The topography of the North Central plains is mostly hilly with some ridges. The soils consist of clays, sands and silt loam.
4. **Delta/Mississippi Alluvial Plain:** this lies between the Mississippi River and the Loess Hills region, characterized by a relatively flat topography. Swamps also characterize the area. The soil is mostly clay, except along streams, where it consists of sandy loam. These soils (termed alluvium) were deposited by the Mississippi River.
5. **Flatwoods:** Topography of Flatwoods region is flat to slightly hilly, with soils of clay and some sand. Many plants found in the Flatwoods are the same species as those of the North Central Plains.

6. **Pontotoc Ridge/Pontotoc Hills:** The topography of Pontotoc ridge consists of hills and ridges with sandy loam soils. The province is predominantly characterized by a pine/oak mixture.
7. **Blackland Prairie:** The Black Prairie region has a gently rolling topography with mostly calcareous, loamy clay soils. Chalk cedar glades are prevalent in the southern portion where *J. virginiana* is the dominant woody vegetation.
8. **Tishomingo Hills/Fall Line Hills:** In the northeastern counties of Fall Line Hills, the topography is characterized by outcrops of limestone, sandstone and shale. Moving south the topography becomes hilly. The soil of this region consists mostly of sand and clay loam. This area represents a transition zone between the Appalachian Mountains and the Gulf Coastal Plain and it contains habitats that harbor some northern and southern species.
9. **Bluff/Loess Hills:** The bluff region consists of relatively narrow strip bordering the eastern edge of the Mississippi Alluvial Plain. The soil in this region consists of wind-deposited, non-stratified, calcareous silt. The woody vegetation is quite diverse.

CHAPTER 3.

BIOPROSPECTION OF *J. VIRGINIANA* FROM NINE PHYSIOGRAPHIC REGIONS OF MISSISSIPPI

3.1 Abstract

J. virginiana L. (Family Cupressaceae) is a widely distributed species in the United States and parts of Canada. It produces two important chemical products, the anticancer compound podophyllotoxin and essential oil. The objective of this study was to evaluate variations in podophyllotoxin concentration in *J. virginiana*, across nine physiographic regions in Mississippi: Tishomingo Hills, Bluff Region, Blackland Prairie, Jackson Prairie, Delta, Pontotoc Ridge, Flatwoods, North Central Plateau, and Piney Woods. The content of podophyllotoxin in needles across different physiographic regions varied from 0.11 % to 0.36 %. This variation was not statistically significant, suggesting that soil nutrient concentrations and topography within Mississippi may not affect the synthesis and the accumulation of podophyllotoxin in *J. virginiana*. Podophyllotoxin accumulation seems to be a stable trait. The results demonstrated that *J. viriginiana* could be used as a consistent source of podophyllotoxin, and the collection of plant material for commercial extraction of podophyllotoxin can be done independent of the site of collection within the state.

3.2 Introduction

There can be an enormous degree of intra-specific variation observed in plants at the morphological, genetic and chemical/physiological levels (Meloni *et al*, 2006; Muzquiz *et al*, 1994; Purohit *et al*, 1999; Roger *et al*, 2008; Wheeler *et al*, 2007). Forty-two neem ecotypes collected from all-over India have shown a wide variation in the content of oil, and their physicochemical characteristics (Kumar and Parmar, 1996). Variation has been observed in the content of artemisinin in plants of *Artemisia annua* L. collected from different geographical origins, proving the existence of chemotypes (Wallaart, 2000). Before this, in 1997, large variability in essential oil composition was reported among different *A. annua* cultivars cultivated in Finland (Holm *et al*, 1997). Difference in location, lead to significant difference in the the polyphenol content of plants as well season and stalk position were limiting factors in a cultivar study of tobacco (Williamson and Gwynn, 1982). Difference in podophyllotoxin concentration was observed in different populations of Indian mayapple (*Podophyllum hexandrum* Royle) (Purohit *et al*, 1999) and different accessions of American mayapple (*Podophyllum peltatum* L.) collected from various locations (Bedir *et al*, 2002).

Study of such variation is important in order to standardize an industrial process. For instance, the level of polyphenols in tobacco affects the composition of smoke, thus affecting the quality of tobacco (Williamson and Gwynn, 1982). Variations in chemical profile within a species could lead to variability in bioactivity of plant extracts. For example, insect growth inhibition of *Spodoptera litura* Fabricius revealed a wide variation in the half maximal effective concentration (EC_{50}) of the oils of *Azadirachta indica* A. Juss. (Kumar and Parmar, 1996).

Standard methods of isolation of active constituents may lead to variable yields of the pure compound due to differences in the plant material source. Study of these variations and factors affecting variability in a specific area is termed as bioprospection. Bioprospection is commonly used in order to determine the highest yielding accessions/cultivars that could be used by the pharmaceutical industry. The present chapter discusses the bioprospection (or study of variation) of an anticancer compound podophyllotoxin (Figure 5) from *J. virginiana* L. (Figure 1) (Family Cupressaceae) in nine physiographic regions in Mississippi (Figure 6). Earlier studies on *J. virginiana* showed clinical variation based on terpenoid data from different populations collected along transect from northeast Texas to Washington DC (Flake *et al*, 2007). Nine populations collected along this 1500 miles show a clustering of these populations clinically from northwest to southwest. In another study significant difference was observed in the composition of leaf essential oil obtained from Texas and Ontario accessions (Semen and Hiziroglu, 2005), indicating a remarkable geographic affect. Age of the tree was also proved to be detrimental in the composition of essential oil by Dunford *et al*, 2006. Significant changes in leaf oil concentration and profile of *J. virginiana* was found between sexes and between habitats within a single geographic region (Setzer *et al*, 1992). Changes in podophyllotoxin concentration were observed in *J. virginiana* with regard to the maturity, leaf stage, and sampling date (Cushman *et al*, 2003). However, none of these works considered studying variation across different locations for *J. virginiana* with respect to the amount of podophyllotoxin. Considering the lack of previous research and the fact that Mississippi has diverse soil types, climatic characteristics, and topography (Stewart, 2003), the present study was conducted to

determine variation in podophyllotoxin content of *J. virginiana* from nine physiographic regions in Mississippi.

Podophyllotoxin is commonly found in *Podophyllum* spp. i.e. Indian/Himalayan mayapple (*Podophyllum emodi* Wall. ex Royle (syn. *P. hexandrum*) and American mayapple (*Podophyllum peltatum* L.). Indian mayapple which yields the highest amount of podophyllotoxin and grows wild in India and other countries in the Himalayan region, is currently the only source for podophyllotoxin (Bohlin and Rosen, 1998). However, Indian mayapple has been declared endangered (Foster, 1993; Rai *et al*, 2000). American mayapple could be an alternative domestic source for podophyllotoxin, although the content is relatively low compared to Indian mayapple. American mayapple grows only in the spring and requires stringent conditions of growth. It is a lower canopy plant and yields only 2-3 leaves per plant per growing season. These challenges have so far prevented the introduction of American mayapple as a viable crop for podophyllotoxin production. The alternative domestic source of podophyllotoxin to both of these species is *J. virginiana* L., which grows widely in the United States and Canada. In some states, this species has been declared invasive and management strategies have been adopted to control it (Gold *et al*, 2005). Although the content of podophyllotoxin is higher in mayapple than in *J. virginiana*, the advantages like round the year availability and large amount of biomass makes the latter a better candidate for commercial utilization of podophyllotoxin. Eastern red cedar is widely used in the timber industry with a $60 million annual market in 2005 (Gold *et al*, 2005). Leaves (needles) are a major by-product of this industry.

Mississippi is known to show diversity in climatic factors, soil type, topography and vegetation from north to south and east to west. The state is broadly divided into nine physiographic regions. Considering this geographic variation the chemo-variation in these physiographic regions becomes an essential step in utilization of *J. virginiana* as an alternate source of podophyllotoxin. The objective of this study was to evaluate variations in podophyllotoxin concentration in *J. virginiana* leaves (needles) across nine physiographic regions in Mississippi: Tishomingo Hills, Bluff Region, Blackland Prairie, Jackson Prairie, Delta, Pontotoc Ridge, Flatwoods, North Central Plateau, and Piney Woods.

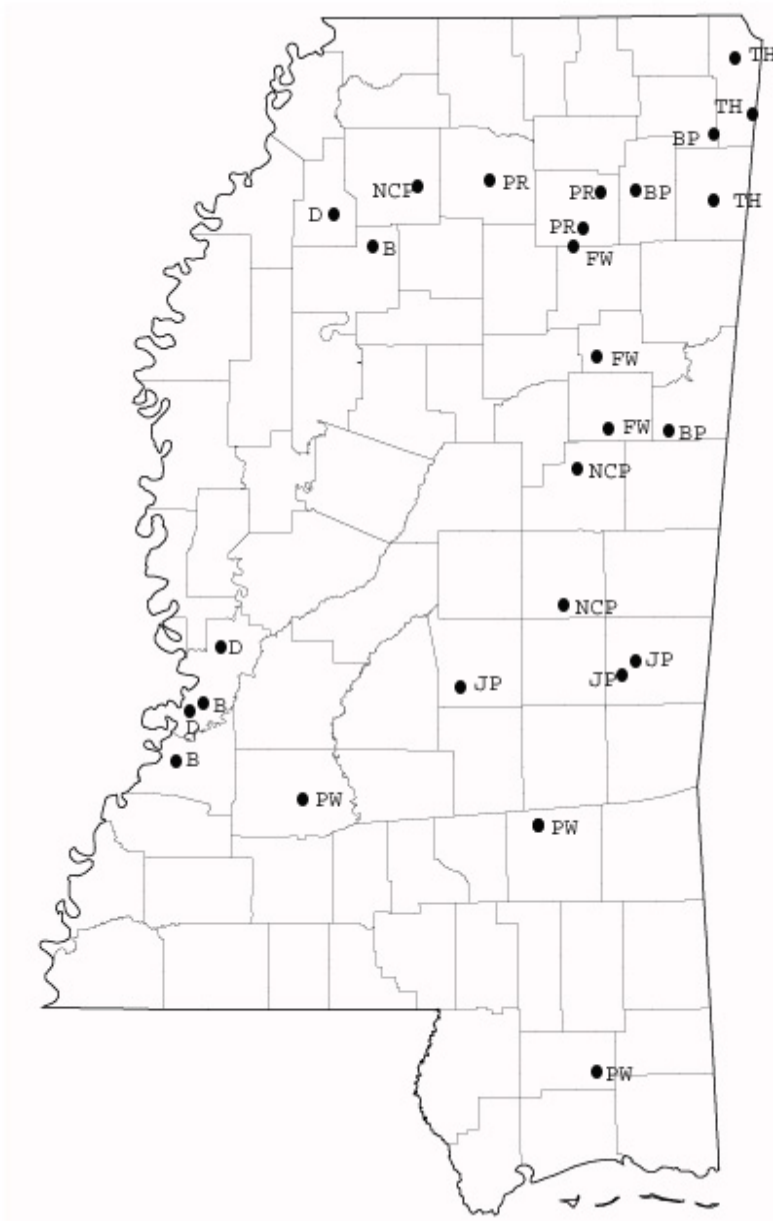


Figure 6 Locations for collections of *J. virginiana* in nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney Woods

3.3 Material and Methods

3.3.1 Collection of Plant Material

Bioprospection of *J. virginiana* was conducted across nine physiographic regions of the state of Mississippi in March 2007 and then repeated in March 2008 (Table 1 and Figure 6). These physiographic regions were selected considering soil type and elevation. The nine physiographic regions are: Delta (D), Bluff Region (B), Jackson Prairie (JP), Blackland Prairie (BP), Tishomingo Hills (TH), Pontotoc Ridge (PR), North Central Plateau (NCP), Piney Woods (PW) and Flatwoods (FW). Three accessions were obtained per physiographic region from different locations all spread across the respective region, rather than restricting to a single site in a region. The name of the collector, place of collection (county, state), date of collection and the respective physiographic region were recorded. The data included altitude and co-ordinates (latitude, longitude) for the collection site recorded using a Global Positioning System (GPS) Garmin 276C (Garmin Ltd., Olathe, KS). Every location was thus identified by the GPS waypoint number, in addition to the name of the county. Herbarium samples were assigned a voucher number that served as the accession number for each plant collected. A digital picture was also taken for a digital library. Other data recorded included plant height, which was calculated using an electronic clinometer (Haglof, Sweden), girth of the tree, sex (this was concluded on the basis of presence of yellow to brown colored cones in males and blue-gray colored cones/berries in females). The data on the frequency of occurrence of the species was recorded in 5 categories: (1) abundant; (2) common; (3) frequent; (4) uncommon; and (5) rare. The determination of occurrence was done by visual

observation and no numerical data on species density per unit area was collected while assigning the above mentioned categories. The category abundant was assigned for those regions where large extended patches of *J. virginiana* were observed across the region, whereas rare category was assigned to those regions where very few plants were found or where plants were difficult to locate for collection. Information on associated species was also identified and recorded. Approximately 1 kg of fresh leaves (needles) was collected from three levels (i.e. lower, middle and top) from around the tree using hand and/or tree pruners. These were placed in large brown paper bags. Soil samples (4-5 cores per site, under the tree canopies, and at 0-15 cm depth) were collected and placed in small paper bags. The plant material was dried in an oven at 40 ^{o}C for 72 hours.

3.3.2 Soil Analysis

The soil samples were tested for soil pH, phyto-available nutrients, total soluble salts and organic matter at the Mississippi State University Extension Service, Soil Testing Laboratory, using commonly accepted methods (Cox, 2001).

3.3.3 Chemical Analysis

The oven dried plant material was weighed, ground with a grinder (Magic Bullet®) and used for extractions and measurements of podophyllotoxin with High Performance Liquid Chromatography (HPLC) (Canel *et al*, 2001) (Figure 7, 8).

2.3.4. Extraction and Analysis of Podophyllotoxin

40 mg of dried plant material was dissolved in 0.6 mL of phosphate buffer (pH 7) and incubated on an Eppendorf Thermomixer for 30 min at 800 rpm. To every sample, 0.8 mL ethyl acetate was added and incubated for another 10 minutes. The ethyl acetate phase was separated by centrifugation (Savant speed vac. svc 200), and was collected and dried under a stream of nitrogen. The residue was re-dissolved in HPLC grade methanol and used for HPLC analysis (Canel *et al*, 2001). Extracts were analyzed using isocratic HPLC for 20 min with a flow rate of 1 mL/min, followed by a 10 min methanol wash and a re-equilibration for 5 min. The instrument was an Agilent (Agilent, Palo Alto, CA) 1200 series consisting of a degasser, fitted with a quaternary pump, ALS autosampler, diode array detector, and Agilent Eclipse XDB-C18 4.6x150 mm, 5 μm column. The injection volume was 10 μL. All sample injections were analyzed at room temperature. The mobile phase was a 28:72 mixture of acetonitrile:water, [0.025 % trifluoroacetic acid (TFA)], and monitoring was done at 220 nm. (Canel *et al,* 2001). A standard curve was obtained with a reference standard of podophyllotoxin (0.01 mg/L-1mg/L). Quantitative analysis was performed using the software generated calibration curves (R^2=0.9999). All chemicals used for HPLC analysis were HPLC grade from Fisher Scientific (Waltham, MA).

3.3.5 Statistical Analysis

Analysis of variance was conducted on the data in order to determine if there is variation in podophyllotoxin content in the nine physiographic regions and the soil elements, separately.

3.4 Results and Discussion

The concentration of phyto-available soil nutrients and pH in the soils collected from the nine physiographic regions are summarized in Table 4. The concentrations of P (coefficient of variance= 0.0102), Ca (coefficient of variance= 0.0061), and Mg (coefficient of variance=0.0004) in soils show variation along the physiographic regions. Also, there were large variations in soil pH (coefficient of variance =0.0022) among the regions (Table 4, Figure 11-18).

The data collected on associate species (Table 2) indicated that the most common associate species were *Lonicera japonica* Thunb. (in 56% of the locations), *Pinus taeda* L. (in 63% of the locations), *Quercus nigra* L. (in 41% of the locations) and *Smilax bona-nox* L. (in 26% of the locations) (Table 2). Various growth forms like dwarf-conical, oval, and spread-types to elongated conical forms were observed in *J. virginiana*. In addition, there was a variation in leaf color; from dark green, olive green, bluish green to very light green shades (Table 1). The height and the girth of the trees varied mainly because of the different growth forms observed in the species (data not shown). Additionally, the height might have varied because of difference in age of the plants.

Earlier studies on variation of podophyllotoxin in *J. virginiana* were done with regard to the maturity, stage of leaf, and sampling date (Cushman et al., 2003). It was observed that matured leaves (needles) show higher podophyllotoxin content than juvenile needles and the needles harvested in Jan and Apr yield higher amounts of podophyllotoxin than those harvested in the month of February and June (Cushman *et al*, 2003). In the present study bioprospection in different physiographic regions was conducted in order to find the best potential region(s) as a source of podophyllotoxin

from *J. virginiana*. The results did not indicate significant differences between the nine physiographic regions in leaf podophyllotoxin concentrations in either of the collection times (March 2007 and March 2008) (Table 3, Figure 9 and 10). There was no difference concentration of podophyllotoxin in male/female trees with an average concentration of 0.179%DW in male and 0.221% DW in female during the first collection and an average concentration of 0.202% DW in male and 0.209% in female during the second collection.

The study of correlation of the soil properties and podophyllotoxin show that the podophyllotoxin content during the second year shows correlation with % organic matter (co-efficient of correlation = 0.74452, p-value=0.0214), potassium (coefficient of correlation= 0.96577, p-value =<0.0001) and magnesium (0.67159, p-value=0.0476). However, the content of podophyllotoxin for the first year lacks correlation with any of the soil properties. The correlation value of podophyllotoxin between the two years also varies with a coefficient correlation of 0.57209, which explains why the correlation of podophyllotoxin for 1st year with soil elements can be different from that during the second year. However, the reason between the differences in the content of podophyllotoxin in the same trees for two consecutive years is difficult to find. Out of the many possible reasons, the pruning of the trees during the first year might have increased the content in the second year, since plants are known to react to wounding injury by enhancement of secondary metabolites. Other reasons could be a difference in climates during the two years i.e. 2007 and 2008.

These findings suggest that podophyllotoxin concentration in *J. virginiana* is a stable trait. Lack of variation in the concentration of podophyllotoxin across physiographic regions means no regional specificity for collection of *J. viriginiana* as a

source of podophyllotoxin. Thus *J. virginiana* could provide a consistent source for podophyllotoxin, unlike American mayapple (*Podopyllum peltatum*) in which the podophyllotoxin concentration was found to have a wide range, with some chemotypes lacking any podophyllotoxin (Bedir *et al*, 2002).

3.5 Conclusions

From this work it has been demonstrated that location (physiographic region), soil phyto-available nutrients, soil pH, the type and occurrence of associated species, and the morphological features do not affect the synthesis and accumulation of podophyllotoxin in *J. virginiana*. The concentration of podophyllotoxin in *J. virginiana* is constant across the state of Mississippi. Hence, *J. virginiana* leaves (needles) could be a dependable source for podophyllotoxin in Mississippi.

Table 1 Collection data of *Juniperus virginiana* for nine-physiographic regions of the state of Mississippi

Sr. no	Voucher No.	County	Physio graphic Region	GPS ref no.	Latitude	Longitude	Altitude (m)	occurrence	Leaf color code	male/ female
1	4846	Prentiss	BP[z]	5293	34 N 40.101	88 W 35.970	150.27	****	4GY4/8	M
2	4864	Lee	BP	5418	34 N 18.310	88 W 45.856	92.53	*****	5GY4/6	M
3	4868	Lowndes	BP	5441	33 N 18.105	88 W 36.168	78.13	*****	5GY4/6	M
4	4862	Itawamba	TH	5400	34 N 13.941	88 W 20.498	363	****	5GY4/6	F
5	4863	Tishomingo	TH	5417	34 N 36.295	88 W 13.216	630	**	5GY3/4	M
6	4848	Tishomingo	TH	5297	34 N 47.842	88 W 13.234	609	***	5GY4/8	M
7	4865	Lee	PR	5422	34 N 19.755	88 W 53.875	467	****	5GY4/8	M
8	4866	Pontotoc	PR	5435	34 N 09.870	89 W 00.666	452	****	5GY4/6	F
9	4849	Lafayette	PR	5353	34 N 21.82	89 W 30.349	471	***	5GY4/6	F
10	4854	Winston	NCP	5367	33 N 10.226	89 W 02.946	517	**	5GY4/6	M
11	4874	Neshoba	NCP	5555	32 N 35.601	89 W 06.603	570	****	5GY4/8	M
12	4850	Panola	NCP	5354	34 N 19.489	89 W 49.755	309	****	5GY4/8	M
13	4861	Warren	D	5399	32 N 28.371	90 W 48.562	122	*	5GY4/8	M
14	4859	Warren	D	5397	32 N 11.800	90 W 56. 705	90	*	5GY4/8	M
15	4851	Quitman	D	5360	34 N 12.257	90 W 14.844	161	*	5GY4/8	M
16	4857	Claiborne	B	5395	32 N 00.620	91 W 02.173	146	****	5GY4/6	F
17	4860	Warren	B	5398	32 N 12.238	90 W 56.590	173	**	5GY4/6	M
18	4852	Tallahatchie	B	5363	34 N 06.352	90 W 04.042	334	***	5GY4/6	F
19	4853	Oktibbeha	FW	5366	33 N 18.535	88 W 54.506	262	*	5GY4/6	F
20	4867	Pontotoc	FW	5436	34 N 05.513	89 W 02.728	383	**	5GY4/8	F

Table 1 (continued)

Sr. no	Voucher No.	County	Physiographic Region	GPS ref no.	Latitude	Longitude	Altitude (m)	occurrence	Leaf color code	male/ female
21	4875	clay	FW	5569	33 N 35.475	89 W 00.594	331	**	5GY4/8	M
22	4871	Lauderdale	JP	5544	32 N 20.823	88 W 51.882	398	****	5GY4/8	M
23	4872	Lauderdale	JP	5545	32 N 21.240	88 W 49.543	368	****	5GY4/8	M
24	4855	Scott	JP	5371	32 N 17.994	89 W 36.945	456	****	5GY4/4	M
25	4845	Jackson	PW	5292	30 N 43.549	88 W 55.060	4.3	**	5GY4/8	M
26	4856	Copiah	PW	5373	31 N 49.091	90 W 25.851	326	****	5GY4/8	M
27	4873	Jones	PW	5546	31 N 46.071	89 W 15.706	374	**	5GY4/8	F

[z]BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods.

*****: Abundant, ****: common, ***: frequent, ** uncommon, * rare

M: Male, F: Female

Table 2 Associate species of *Junipers virginiana* in nine physiographic regions of Mississippi

		BP			TH			PR			NCP			D			B			FW			JP			PW	
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Acer negundo													*[z]	*										*	*		
Acer rubrum													*														
Albizia julibrissin										*																	
Ambrosia artemisiifolia															*												
Ampelopsis cordata													*														
Andropogon glomeratus			*																								
Andropogon virginicus		*						*		*								*	*								
Aralia spinosa										*																	
Arundinaria gigantia										*											*				*		
Asplenium platypleura																								*			
Baccharis halimifolia																									*		
Bignonia capreolata												*															

Table 2 (continued)

		BP			TH			PR			NCP			D			B				FW			JP			PW			
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Campsis radicans													*														
Carex cherokeensis	*																										
Carya glabra																	*										
Celtis laevigata																											
Cercis canadensis		*																									
Chasmanthium laxiflorum														*										*			
Chrysopsis grandifolia																	*										
Cornus florida							*																				
Cynodon dactylon	*					*			*																		
Dichantheliu m laxifolia																		*						*			*
Elymus virginicus																								*			
Eupatorium capillifolium																									*		

Table 2 (continued)

			BP			TH			PR			NCP			D			B			FW			JP			PW	
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Gleditsia triacanthos	*																										
Lespedeza cuneata								*																			
Ligustrum snorse																								*			
Liriodendron tulipifera										*																	
Liquidambar styraciflua										*														*			
Lolium arundinaceum	*		*																								
Lonicera japonica	*	*	*		*					*	*			*	*	*	*			*	*	*		*		*	
Myrica cerifera																									*		
Nyssa sylvatica							*																				
Ostrya virginiana										*								*									
Persia palustris																									*		
Pinus echinata						*																					

Table 2 (continued)

		BP			TH			PR			NCP			D			B			FW			JP			PW		
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Prunus serotina								*	*															*	*			
Pteridium aquilinum																					*							
Quercus niagra					*		*							*	*		*	*		*				*		*	*	*
Quercus alba						*															*							
Quercus falcata							*																					
Quercus pagoda					*										*		*	*								*		
Quercus stellata																*												
Quercus virginiana																									*			
Rubus trivialis	*												*		*							*						
Saccharum giganteum																	*											
Scleria oligantha										*						*												
Smilax bona nox			*								*							*		*				*	*	*		
Smilax glauca					*		*																					

Table 2 (continued)

		BP			TH			PR			NCP			D			B			FW			JP			PW	
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Smilax rotundifolia																					*	*			*		*
Smilax snakii																									*		
Solidago canadensis	*																										
Sorghum halepense	*	*													*												
Stellaria media												*															
Tillandsia usneoides																									*	*	
Toxicodendron radicans															*												
Triadica sebifera																									*		
Tridens flavus								*																			
Trifolium incarnatum	*																										
Ulmus alata							*	*		*														*			
Ulmus americana																	*										
Ulmus rubra														*			*										
Vaccinium elliottii																			*			*	*				

Table 2 (continued)

		BP			TH			PR			NCP			D				B			FW			JP				PW	
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Vaccinium staminenum																		*	*	*							
Verbena brasiliensis																											*
Vitex rotundifolia					*												*										
Wrigstrum sinensis	*																										

[z]* indicates presence of the species as an associate of the respective accession of *Juniperus virginiana*.

[y]BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney Woods.

Table 3 Concentration of podophyllotoxin in the leaves (needles) of *J. virginiana* collected in the state of Mississippi

Sr. No	Physiographic Region	2007 PDP[z] (%)	2007 Variance	2008 PDP (%)	2008 Variance
1	BP[y]	0.2657a[x]	0.0142	0.2395a	0.0246
2	TH	0.1470a	0.0286	0.1820a	0.0676
3	PR	0.2453a	0.0687	0.1851a	0.0480
4	NCP	0.2087a	0.0844	0.1778a	0.0737
5	D	0.1500a	0.0210	0.2754a	0.0225
6	B	0.2907a	0.1045	0.3386a	0.1395
7	FW	0.1947a	0.0113	0.1478a	0.0157
8	JP	0.1097a	0.0319	0.1495a	0.0459
9	PW	0.1283a	0.0781	0.1693a	0.0777

[z]PDP - Podophyllotoxin

[y]BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney Woods.

[x]Values within a column followed by same letter are not significantly different at P 0.05.

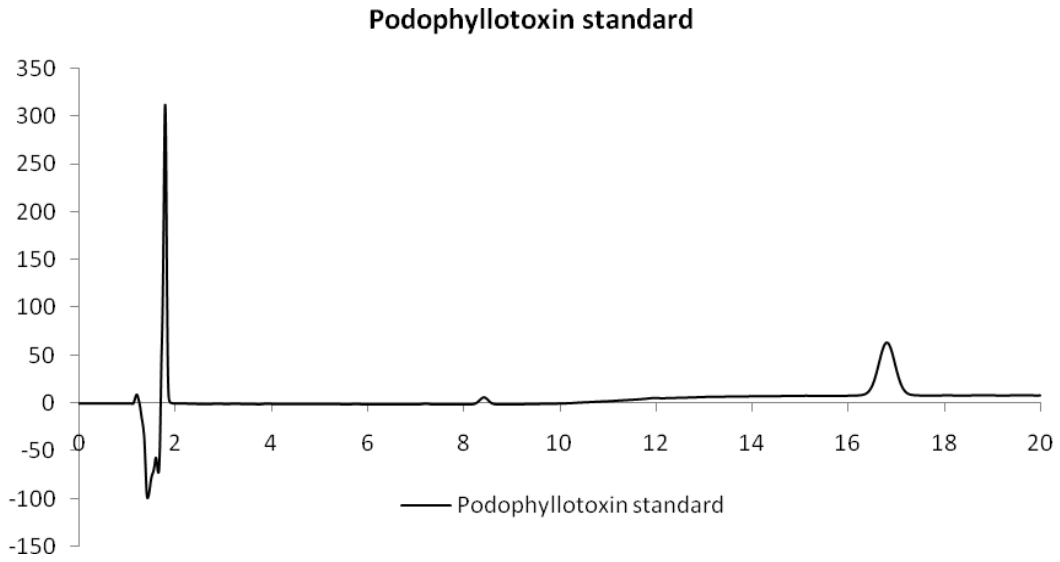


Figure 7 HPLC detection of podophyllotoxin reference standard at 220 nm

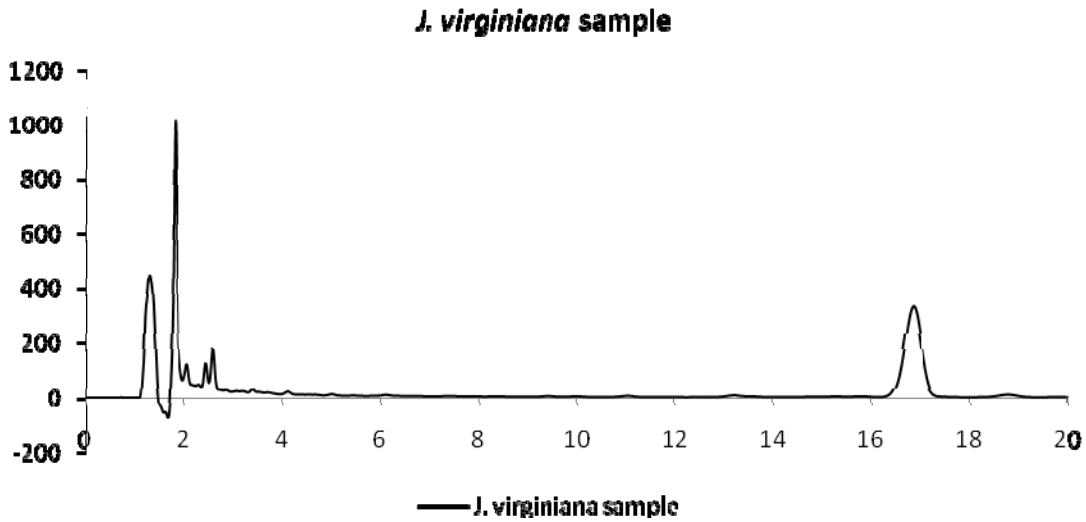


Figure 8 HPLC detection of *J. virginiana* sample extract at 220 nm

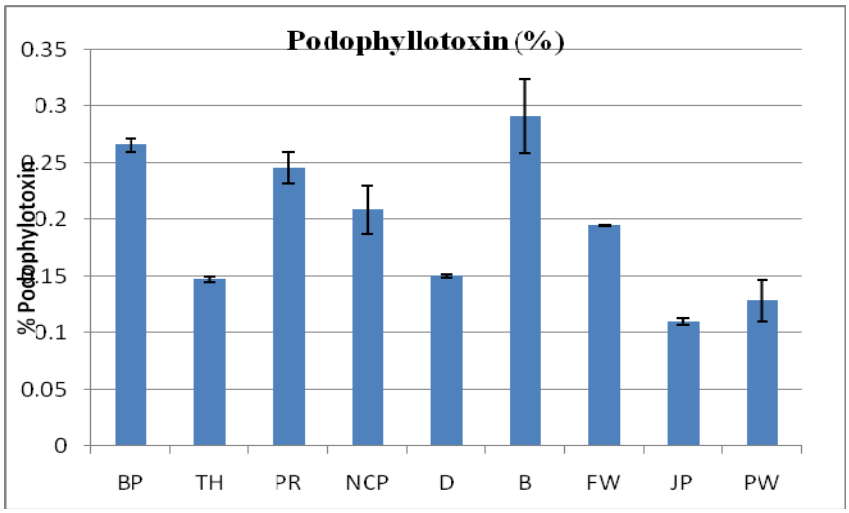


Figure 9 Podophyllotoxin content in accessions from nine physiographic regions for the 1st collection (2007).

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

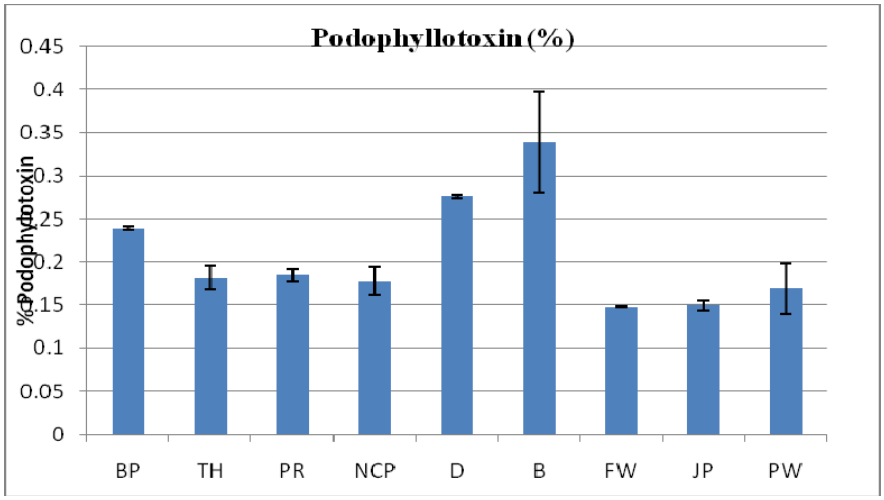


Figure 10 Podophyllotoxin content in accessions from nine physiographic regions for the 2nd collection (2008).

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

Table 4 Soil analysis data for the locations of collection of *J. virginiana* from nine physiographic regions of Mississippi

Sr. No	Physiographic region	% OM	pH	TSS	P	K	Ca	Mg	Zn	Na
1	BP[z]	3.17a[y][x]	7.74a	2a	33b	290a	8630a	265d	2.4a	105a
2	TH	2.9a	5.7bc	1a	22b	220a	2258bc	269d	2.2a	74a
3	PR	1.83a	5.3c	1.3a	23b	250a	1760c	300d	2.7a	77a
4	NCP	1.93a	4.9c	1a	19b	229a	1763c	748bc	3.7a	85a
5	D	3.8a	6.6ba	1.53a	107a	384a	5341ba	1299a	21a	101a
6	B	3.97a	6.0bc	1.3a	105a	432a	4074bc	1101ba	6.9a	87a
7	FW	2.43a	4.9c	1.13a	59ba	229a	2396bc	654dc	4.8a	297a
8	JP	2.67a	4.9c	1a	22b	168a	2672bc	358dc	4.7a	83a
9	PW	3.33a	5.3c	1a	69a	149a	1186c	286d	5.2a	118a

[z]BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods,

JP: Jackson Prairie, PW: Piney woods.

[y]Mean values with same letters are not significantly different at P 0.05

[x]P value for % OM = 0.1413, P value for pH = 0.0022, P value for P (phosphorus) = 0.0102, P value for K = 0.0954, P value for Ca = 0.0061, P value for Mg = 0.0004, P value for Zn = 0.3109, P value for lime = 0.1630, P value for Na = 0.4062

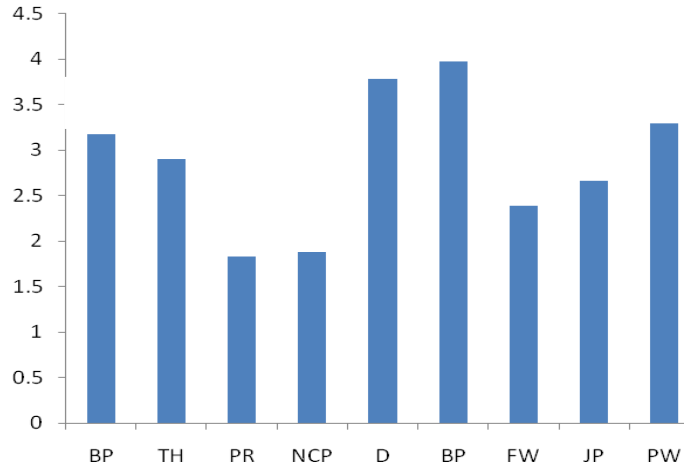


Figure 11 % Organic matter in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

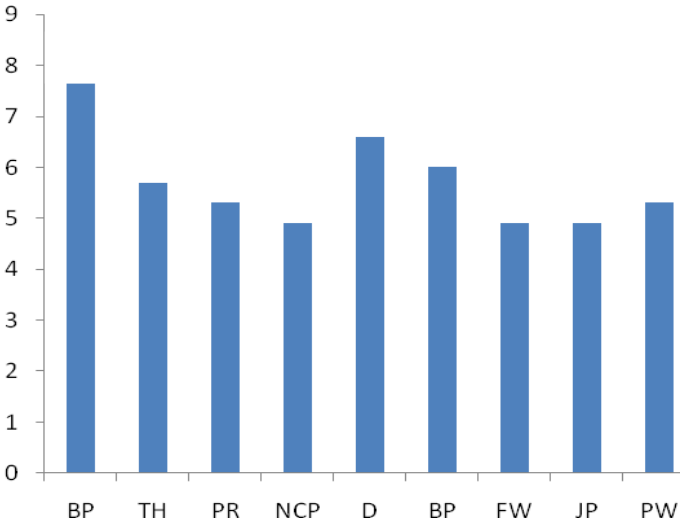


Figure 12 pH of soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

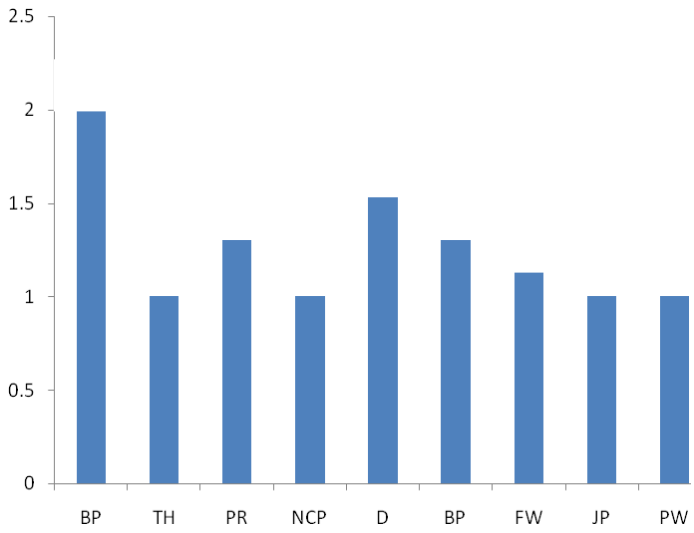


Figure 13 TSS in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

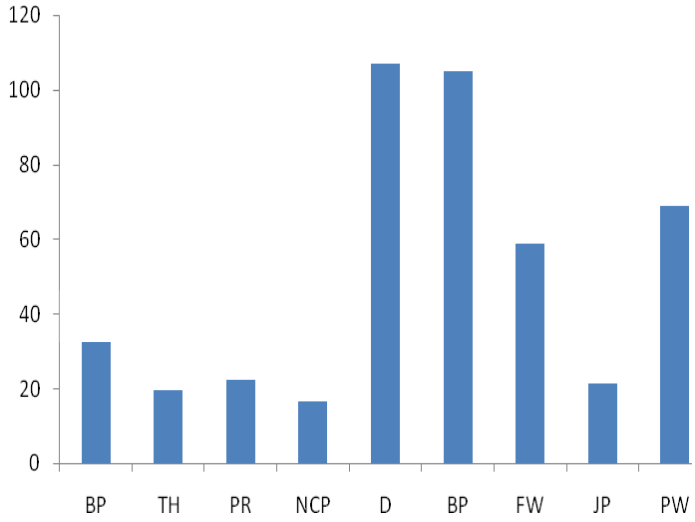


Figure 14 P content in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

Figure 15 K content in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

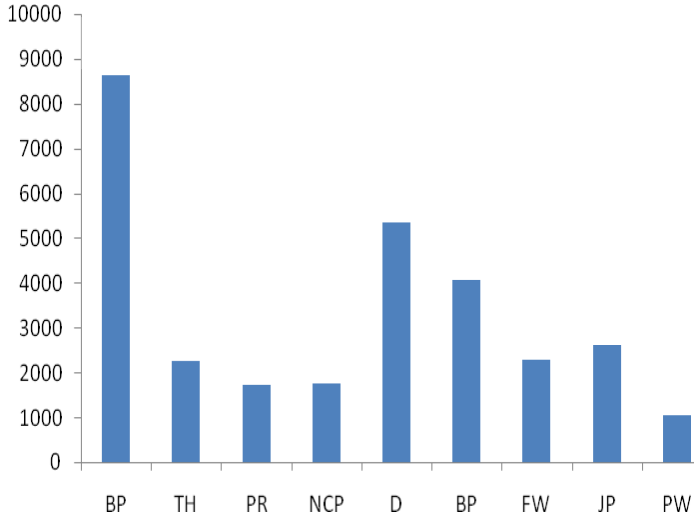


Figure 16 Ca content in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

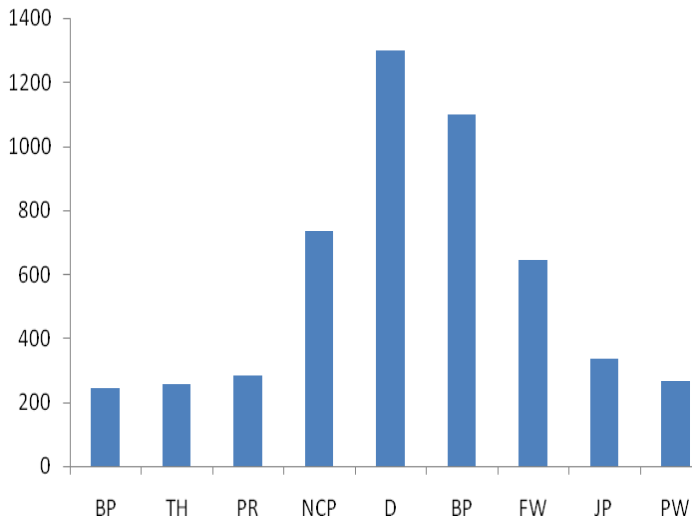


Figure 17 Mg content in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

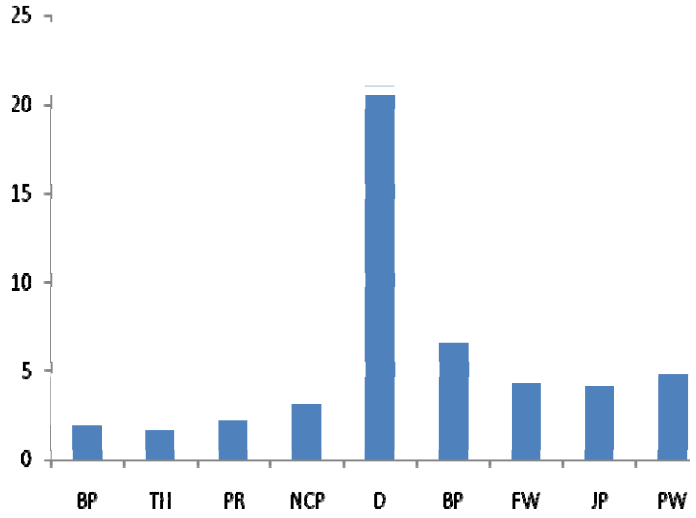


Figure 18 Zn content in soils from nine physiographic regions of Mississippi

BP: Blackland prairie, TH: Tishomingo Hills, PR: Pontotoc Ridge, NCP: North Central Plateau, D: Delta, B: Bluff, FW: Flatwoods, JP: Jackson Prairie, PW: Piney woods

CHAPTER 4.

BY-PRODUCT UTILIZATION AND DUAL EXTRACTION OF PODOPHYLLOTOXIN AND ESSENTIAL OIL

4.1 Abstract

The needles of Eastern red cedar (*J. virginiana* L.) contain two important natural products: essential oil and podophyllotoxin. The hypothesis of this study was that it may be possible to extract both essential oil and podophyllotoxin from the needles of the tree, by using a dual extraction method. Podophyllotoxin was obtained from the needles after steam distillation of the essential oil, indicating that steam distillation did not degrade podophyllotoxin. Furthermore, steam distillation improved the extraction of podophyllotoxin compared to the extraction of podophyllotoxin from un-distilled needles. A product with 6 % purity of podophyllotoxin was obtained from the distilled plant material, thus establishing an industrially economic protocol for dual extraction of these two natural products. Our study demonstrated that *J. virginiana* needles, a waste-product from the timber industry, could be sequentially extracted for essential oil and podophyllotoxin and utilized as a by-product instead. We also found that the heartwood (a traditional source of cedarwood oil) does not contain podophyllotoxin.

4.2 Introduction

J. virginiana, well-known for its use of durable, termite resistant and insect resistant heartwood (redwood), has been studied extensively for the composition and yield of essential oil, commonly called cedarwood oil (Coleman and Lawrence, 1997; Payne *et al,* 1999; Eller and King, 2000; Dunford *et al*, 2006). *J. virginiana* needles contain podophyllotoxin, a precursor lignan for anticancer compounds (Cushman *et al*, 2003). Pharmaceutical companies obtain podophyllotoxin from Indian mayapple *Podophyllum emodi* Wall*, (*synonym *Podophyllum hexandrum*), considered endangered now. American Mayapple (*Podophyllum peltatum*) has now been considered an alternate source of podophyllotoxin. Although podophyllotoxin concentration in American mayapple is higher than in *J. virginiana, J. virginiana* may be a more economically and environmentally sustainable source for the compound because of (1) higher biomass production; (2) wider distribution; (3) less complicated cultivation techniques and wider adaptability (if grown as a crop) and (4) possibility to be used year round, being an evergreen.

Plant parts like leaves, bark and sapwood are waste products of the timber industry. Leaves are a good source of podophyllotoxin; the other plant parts have not been explored as podophyllotoxin source. Leaves and bark also produce essential oil, the composition of the latter has not been reported although, the leaf essential oil has been studied for oil composition and yield (Semen and Hiziroglu, 2005; Dunford *et al*, 2006). The present study explores different plant parts of *J. virginiana* as prospective sources of podophyllotoxin. Red cedar needles are thus a good source of two commercially valuable natural products; podophyllotoxin and essential oil. There are no reports on obtaining

both essential oil and podophyllotoxin from the leaves (needles) of *J. virginiana.* The dual extraction of essential oil and podophyllotoxin from the *J. virginiana* needles may be possible, simultaneously or sequentially. Further we hypothesized that during steam distillation, if podophyllotoxin is not degraded, or perhaps is extracted in the water used for distillation, then it is easy to recover podophyllotoxin from the distilled plant material and/or residual water. In addition, this chapter thus discusses the attempt of dual extraction procedure for essential oil and podophyllotoxin from *J. virginiana* needles.

4.3 Material and Methods:

4.3.1 Collection of Plant material

The plant material for the experiments was obtained from *J. virginiana* trees found at the North Mississippi Research and Extension Cen., Verona, MS. Samples from 3 different trees were collected and one sample each of the needles was made for steam distillation. Samples of bark, heartwood and sapwood were collected from the same trees. Bark was obtained by strapping a small amount laterally off the tree. The sapwood and heartwood was drilled as shavings using a wood bit.

4.3.2 Drying Processing and distillation

The plant material (needles on branches not thicker than 3 mm) was dried in an oven at 40^{o} C for 48 hours. Bark, sapwood, heartwood were dried similarly. After that, the plant material was chopped in small pieces of about 1.5 cm long and 400 g of the dried material was steam distillated in a 2L steam distillation unit for 90 min (Figure 19).

The essential oil was collected and stored under refrigeration. The residual plant material (distilled) was retrieved and dried in an oven at 40 ^{o} C for 48 hours in paper bags. The volume of the residual water from distillation was recorded and was stored under refrigeration to be used for analysis of podophyllotoxin. The water that was drained off during oil accumulation i.e. 'hydrolat', was also collected, the volume measured and analyzed for podophyllotoxin. The distilled plant material, un-distilled plant material, bark, sapwood and heartwood were all ground to a fine powder.

4.3.3 Extraction of podophyllotoxin

40 mg each of dried ground plant material (distilled and un-distilled) and bark, sapwood and heartwood was dissolved in 0.6 mL of phosphate buffer (pH 7) and incubated on an eppendorf thermomixer for 30 min at 800 rpm. Then, 0.8 mL of ethyl acetate was added and incubated for another 10 min. The ethyl acetate phase was separated using centrifugation (Savant speed vac. svc 200), collected, and dried under a stream of nitrogen. The residue was re-dissolved in High Performance Liquid Chromatography (HPLC) grade methanol and used for HPLC analysis (Canel *et al*, 2001). Extracts were analyzed using isocratic HPLC for 20 min with a flow rate of 1mL/min, followed by a 10 min methanol wash and a re-equilibration for 5 min.

The volumes of residual water and hydrolat obtained from steam distillation were recorded. 1 mL of these samples were transferred to a HPLC vial and directly injected for podophyllotoxin analysis. In another experiment the residual water and hydrolat were basified using a phosphate buffer (pH 7) and incubated for 30 min with shaking. 1 mL of these samples were transferred to a HPLC vial for analysis of podophyllotoxin.

Essential oil samples were diluted to a 1 mg/mL concentration using methanol and these were used for podophyllotoxin analysis.

4.3.4 Purity and recovery analysis for podophyllotoxin

A box scale experiment was done with 20 g of plant material, both distilled and un-distilled, to determine the purity of podophyllotoxin in ethyl acetate. Samples of 20 g of distilled and un-distilled plant material were basified with 300 mL phosphate buffer (pH 7) and incubated for 30 min with intermittent shaking. 400 mL of ethyl acetate was added and incubated for another 10 min with shaking. The ethyl acetate fraction was separated using a glass vacuum filter. This fraction was evaporated to dryness on a Buchi Rotavap R-114 (Switzerland), equipped with a waterbath B-480 (Neslab-RTE-140). The dried residue was weighed and 3 mg of this residue was dissolved in 1 mL of HPLC grade methanol. This was used for HPLC analysis.

4.3.5 HPLC analysis of podophyllotoxin

Extracts of distilled and un-distilled plant material were analyzed using isocratic HPLC for 20 min with a flow rate of 1 mL/min, followed by a 10 min methanol wash and a re-equilibration for 15 min. The instrument was Agilent 1200 series consisting of a degasser, fitted with quaternary pump, ALS autosampler, diode array detector, and Agilent Eclipse XDB-C18 4.6x150 mm, 5 μm column. The injection volume was 10 μL. All sample injections were analyzed at room temperature. The mobile phase was acetonitrile: water (28:72) 0.025 % trifluoroacetic acid (TFA), λmax=220 nm. A standard

curve was obtained with a reference standard of podophyllotoxin (0.01 mg/L-1mg/L). All chemicals used for HPLC analysis were HPLC grade (Fisher Scientific, Hampton, NH).

4.3.6 Gas-Chromatography and Mass Spectroscopic (GC-MS) analysis of essential oil

Qualitative and quantitative analysis of the leaf essential oil obtained by steam distillation was conducted. The amount of oil (total yield) was expressed (W/W) of essential oil/raw material. Individual concentration gradients for reference standards were prepared for β-pinene, myrcene, linalool, iso-safrole, limonene, safrol and bornyl acetate, to obtain a standard curve for each.

The composition of essential oil was detected by using a Varian Gas Chromatography (GC) equipped with Varian mass spectrophotometer (MS) detector. GC was equipped with a DB-5 column (30 m x 0.25 mm fused silica capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 ºC; column temperature, 60-240 ºC at 3 ºC/min then held at 240 ºC for 5 min; injector temperature 300 ºC; carrier gas, He; injection volume, 1 μL (splitless). MS mass range from 40 to 650 *m/z*, filament delay of 3 min, target TIC of 20,000, a prescan ionization time of 100 msec, an ion trap temperature of 150 ºC, manifold temperature of 60 ºC, and a transfer line temperature of 170 ºC.

The commercial standards were obtained from Sigma Aldrich (St. Louis, MO). Linalool and bornyl-acetate were purchased from Fluka (Switzerland). The oil composition was determined by obtaining individual standard curves for -pinene (R_2= 0.9999), myrcene (R_2= 0.9993), linalool (R_2=0.9991), iso-safrole-(E) (R_2= 0.9998),

iso-safrole-(Z) (R_2= 0.9636), limonene (R_2= 0.9996), safrole (R_2= 0.9995) and bornyl acetate (R_2= 0.9997).

4.4. Results and Discussion

4.4.1 Analysis of podophyllotoxin in bark, sapwood and heartwood

Neither sapwood nor heartwood showed detectable amounts of podophyllotoxin. Only bark showed the presence of podophyllotoxin in very low amounts (0.0006%). The un-distilled leaves contain 0.221 % podophyllotoxin, thus proving to be the best source of podophyllotoxin out of all plant parts of *J. virginiana* (Table 5).

4.4.2 Analysis of podophyllotoxin in distilled products

The amount of podophyllotoxin was estimated in un-distilled plant material, distilled plant material (residual plant material after the distillation of oil), residual water, hydrolat and essential oil (Table 6). Podophyllotoxin was present in both the distilled plant material and in residual water. However, the amount extracted from the water was negligible (0.00076 ± 0.00013% DW). The amount of podophyllotoxin in the distilled plant material was 0.218 ± 0.022 % DW (i.e. 0.626 ± 0.26 g per 400 g of oven dried plant material), which was approximately equal that was detected in un-distilled plant material (0.221 ± 0.014% i.e. 0.686 ± 0.26 g per 400 g of oven dried plant material). This indicates that podophyllotoxin was not degraded during the distillation process. The hydrolat and the essential oil did not indicate presence of podophyllotoxin. (Figure 19 and Figure 20)

4.4.3 Purity and recovery of podophyllotoxin

A box scale experiment was done with 10 g of plant material, both distilled and un-distilled, to determine the purity of podophyllotoxin in ethyl acetate (Table 7). The distilled and un-distilled plant material yielded an ethyl acetate residue of 0.2515±0.015 mg of and 0.0296±0.037 mg respectively. A high purity percentage of podophyllotoxin was obtained i.e. 6.228±0.689 % and 5.8012±1.889 % in distilled and un-distilled respectively.

The total recovery of podophyllotoxin was not significantly different from its recovery from un-distilled plant material; with 67.23 % and 77.76 % in distilled and un-distilled plant material respectively (Table 7).

4.4.4 Essential oil analysis

A total average yield of oil obtained by steam distillation from *J. virginiana* needles was 0.696 ± 0.113 g from 400 g of plant material (0.174 % W/W). This oil was further characterized by quantifying the essential oil components. Since there is no standard essential oil of *J. viriginiana* leaf, only individual components which were commercially available were quantified on basis of past literature. Eight essential oil components (β-pinene, myrcene, linalool, iso-safrole-(E), iso-safrole-(Z), limonene, safrole and bornyl acetate) were quantified using standard GC by obtaining the standard curves for these reference compounds (Table 11).

Safrole was reported as the major active constituent of essential oil of leaves obtained from Texas accessions (Von Rudloff, 1975), whereas that of Ontario had a low concentration of safrole (1.5 %). The essential oil obtained in the present paper (Figure

20, Table 3); shows a high concentration of safrole i.e. 19.012 ± 3.8086 %, closely followed by limonene (18.195 ± 0.6184 %). Limonene was reported to be in high amounts in Ontario accessions of *J. virginiana* leaves with a concentration of 17.8 %, whereas those collected from Texas produced only 0.9 % of limonene. Another study on essential oil obtained from leaves of plant material collected from Oklahoma, reports the presence of 2.6 % limonene (Dunford *et al*, 2006). In the present study the concentration of other components was low as compared to safrole and limonene, with β-pinene (3.041 ± 0.4264 %), myrcene (1.378 ± 0.2128 %), linalool (1.137 ± 0.1505 %), iso-safrole-(E) (0.247± 0.1489 %), iso-safrole-(Z) (0.165 ± 0.0566 %) and the lowest of all bornyl acetate (0.01 ± 0.00372 %). Previous reports found a variation in the content of these components from accessions collected from Texas, Ontario and Oklahoma accessions, with 10.6 % Myrcene and 0.9 % bornyl acetate being reported from Oklahoma accessions (Von Rudloff, 1975; Dunford *et al*, 2006), and 1.4 % and 0.7 % linalool from Texas and Ontario, respectively (Von Rudloff, 1975).

The simultaneous extraction of essential oil and podophyllotoxin, without having to lose any podophyllotoxin during the steam distillation process is perhaps the most important finding of this study. *J. virginiana* is an evergreen and is abundantly available, to an extent that in some states it has been considered invasive (Gold *et al*, 2005). This makes it the most consistent source of both these natural products. In addition, employing the present method of dual extraction a dual benefit can be obtained from the waste material (i.e. needles) of timber industry, a finding that can be used industrially.

In addition to the above benefits, obtaining an approximately 6 % pure product of podophyllotoxin from the distilled red cedar needles is also an important finding. Further

research is needed to purify this product to more than 50 % purity by column separations or precipitation experiments. In addition to the above benefits, obtaining a approximately 6 % pure product for podophyllotoxin from the distilled red cedar needles is also an important finding. Further research is needed to purify this product to more than 50 % purity by column separations or precipitation experiments.

Table 5 Amount of podophyllotoxin in different plant parts of *J. virginiana*

Plant part	**Podophyllotoxin (%DW)**
Bark	0.0006%
Sapwood	Absent
Heartwood	Absent
Leaves/needles	0.221 %

Figure 19 Steam distillation of *J. virginiana* needles

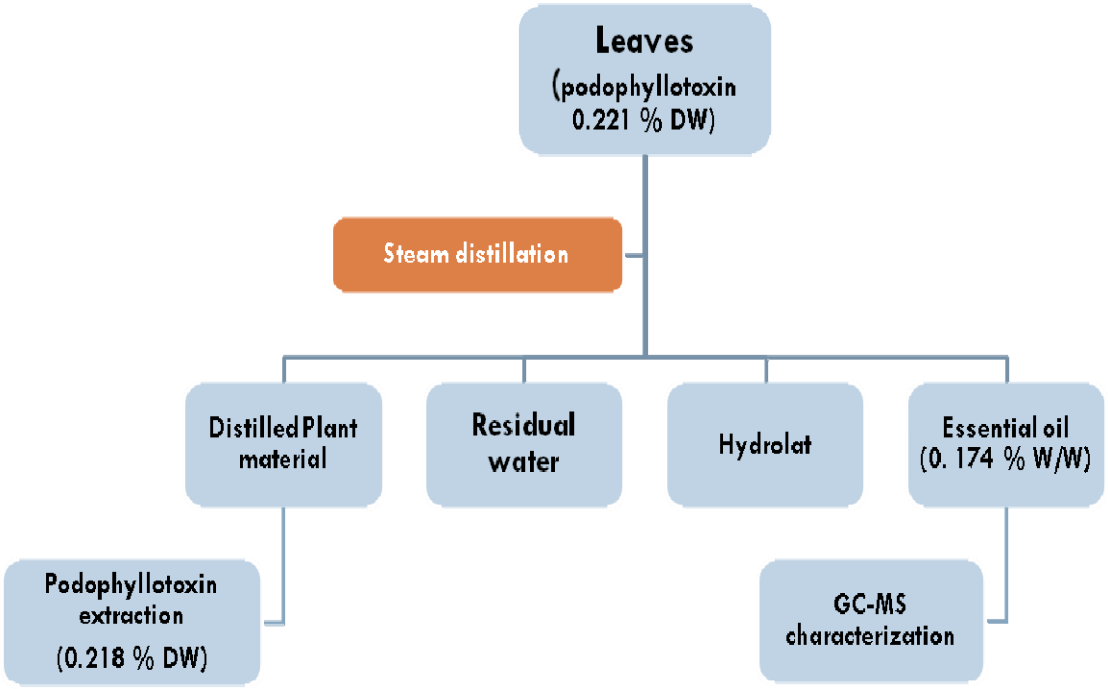


Figure 20 Schematic representation of dual extraction of podophyllotoxin and essential oil from *J. virginiana* leaves

Table 6 Amount of podophyllotoxin obtained from distilled products of *J. virginiana* leaves

Sample	**Podophyllotoxin (% DW)**	**Podophyllotoxin (mg) per 400 g**
Distilled	0.218±0.022	0.626 ± 0.26
Un-distilled	0.221±0.014	0.686 ± 0.26
Residual water	0.00076 ± 0.00013	0.003 ± 0.0005
Essential oil	Absent	Absent
Hydrolat	Absent	Absent

Table 7 Box scale experiments (10 g) for the extraction of podophyllotoxin from un -distilled and distilled plant material

	***PDP (% DW)**	**PDP (g/ 10g DW)**	**Amount of EtoAc residue (g)**	**Purity of podophyllotoxin EtoAc (%)**	**% Recovery**
Distilled	0.156±0.065	0.0156±0.006	0.2515±0.015	6.228±0.689	67 %
Un-distilled	0.172±0.062	0.0171±0.006	0.0296±0.037	5.801±1.889	78 %

*PDP: podophyllotoxin

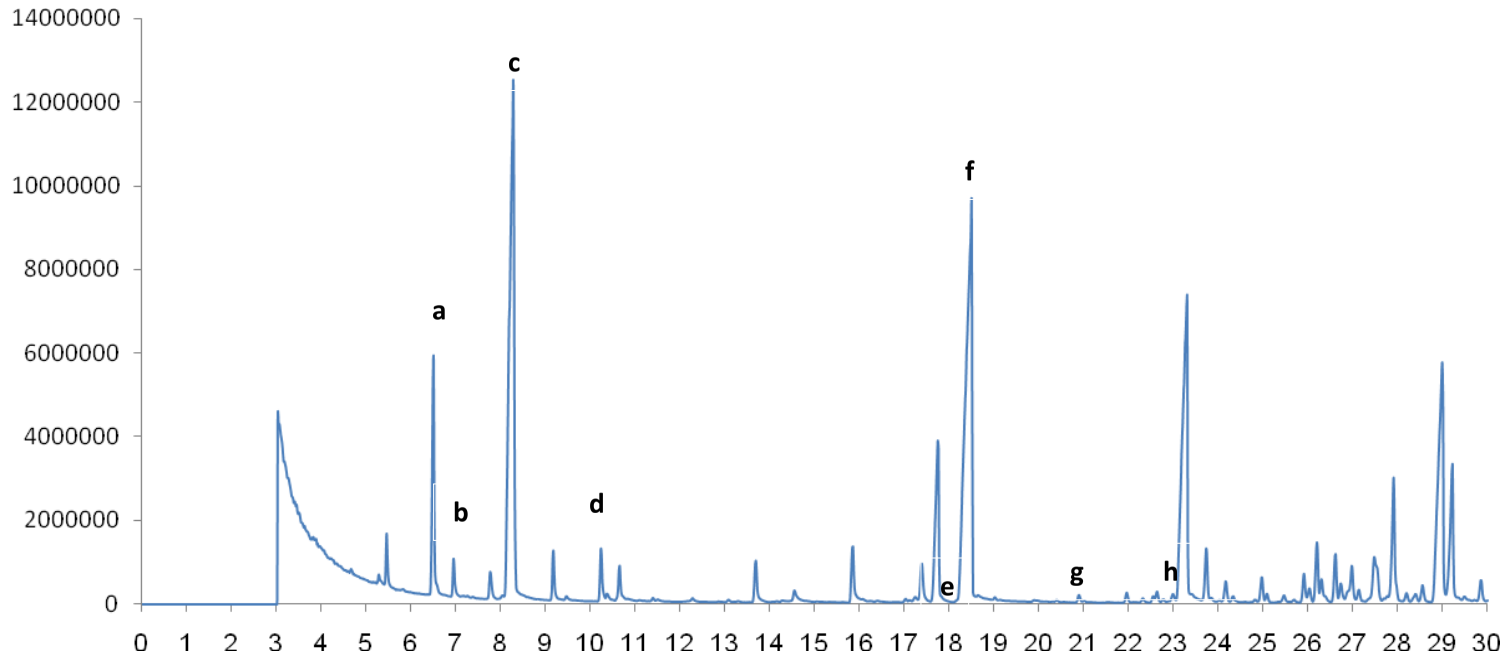


Figure 21 GC-MS profile of essential oil obtained from needles of *J. virginiana.*

a: β-pinene, b: Myrcene, c: Limonene, d: Linalool, e: Bornyl acetate, f: safrole, g: iso-safrole (z), h: iso-safrole (e)

Table 8 Analysis of components of leaf essential oil of *J. virginiana*

Sr. No.	**Compound**	**RT**	**Concentration (%)**
1	Safrole	18.393	19.012 ± 3.8086
2	Limonene	8.141	18.195 ± 0.6184
3	β-pinene	6.568	3.041 ± 0.4264
4	Myrcene	6.902	1.378 ± 0.2128
5	Linalool	10.768	1.137 ± 0.1505
6	Iso-safrole (e)	22.996	0.247± 0.1489
7	Iso-safrole (z)	20.342	0.165 ± 0.0566
8	Bornyl acetate	18.199	0.01 ± 0.00372

RT: Retention Time, Rf: Resolution factor

CHAPTER 5.

SUMMARY

Juniperus virginiana that grows in abundance all over the United States is most commonly used for its timber and cedarwood oil. Tapping the potential of this tree, as a medicinal and aromatic crop for bioprospection of bioactive compounds, was the major aim of this present project.

Results of the present study indicated that the accessions of *J. virginiana* did not show significant variation in the content of podophyllotoxin across the nine physiographic regions in Mississippi that were sampled. Although there was a remarkable variation in the concentration of soil nutrients, diversity of associate species, morphological characteristics and topography, amongst these physiographic regions, these variations may not affect podophyllotoxin. The podophyllotoxin concentration was stable across Mississippi and none of these environmental factors seemed to affect podophyllotoxin synthesis, or they may not be variable large enough to affect synthesis of podophyllotoxin. The site of collection for industrial utilization may be selected on the basis of abundance and access of *Juniperus virginiana,* rather than considering region specificity, since there is consistency in content of podophyllotoxin,

Other plant parts like leaves, sapwood and bark, by-products of the timber industry were explored as potential sources of podophyllotoxin. Other than needles, a

known source, only bark showed podophyllotoxin in low amounts (0.0006% DW), whereas sapwood and heartwood lack any podophyllotoxin. Thus needles remain to be the best source for podophyllotoxin (0.221 % DW). Needles, were also shown to produce essential oil in significant amounts (0.173 % W/W), obtained by steam distillation.

In an attempt to obtain both products, amount of podophyllotoxin was estimated in the steam distilled products i.e. distilled leaves, residual water, hydrolat and essential oil. Distilled plant material (0.218 % DW) and residual water (0.00076 % DW) together produced almost equal podophyllotoxin to un-distilled plant material (0.221 %). Steam distillation did not degrade podophyllotoxin.

In conclusion, *J. virginiana,* a tree with an industry of $ 60 million per year in 2005 throughout the country (Gold *et al,* 2005) proves to be a potential crop for Mississippi for several reasons, in comparison to other sources of podophyllotoxin:

1. Availability of plant material throughout the year
2. Easy cultivation methods
3. Survival capacity and reproductive ability of the tree in diverse climatic conditions
4. Consistency in the content of podophyllotoxin across Mississippi
5. Use of wood in the timber industry
6. Use of heartwood for extraction of cedar-wood oil
7. Use of waste products like leaf as a source of podophyllotoxin and virginiana leaf essential oil

The present research adds invaluable information to the current status of *J. virginiana* in the Medicinal and Aromatic plant industry. The further industrial utilization

and scale-ups of the present projects will definitely need extensive region specific research; however the present study will serve as the basis of the research anticipated in future in this direction.

LITERATURE CITED

1. Bedir, E., I. Khan., and R.M. Moraes. 2002. Bioprospecting for podophyllotoxin. P. 545–549. In: J. Janick and A. Whipkey (eds.). Trends in new crops and new uses. ASHS Press, Alexandria, VA.

2. Beutner K. R. 1996. Podophyllotoxin in the treatment of genital warts. In: E. P. Eischmann (ed.), Sexually Transmitted Diseases: Advances and Treatment. 24: 112-232.

3. Bohlin, L. and B. Rosen. 1998. Podophyllotoxin derivatives: drug discovery and development. Drug Discov. Today. 1(8):343-351.

4. Boehm, R., S. Sommer., S. M. Li., and L. Heide. 2000. Genetic Engineering on Shikonin Biosynthesis: Expression of the Bacterial *ubiA* Gene in *Lithospermum erythrorhizon.* Plant and Cell Physiology, 41 (8): 911-919.

5. Broomhead, A. J., and P. M. Dewick. 1990a. Tumor-inhibitory aryltetralin lignans in *Podophyllum versipelle, Diphylleia cymosa* and *Diphylleia grayi.* Phytochemistry. 29:3831-3837.

6. Broomhead, A. J., and P. M. Dewick. P M 1990b. Aryltetralin lignans from *Linum flavum* and *Linum capitatum.* Phytochemistry. 29:3839-3844.

7. Canel, C., F.E. Dayan., M. Ganzera., I.A. Khan., A. Rimando., C. Burandt, and R.M.Moraes. 2001. High yield of podophyllotoxin from leaves of *Podophyllum peltatum* by i*n situ* conversion of podophyllotoxin 4-O-b-D-glucopyranoside. Planta Med. 67:97-99.

8. Coleman, W. M., and B. M. Lawrence. 1997. A comparison of selected analytical approaches to the analysis of an essential oil. Flavour and Fragrance Journal. 12:1-8.

9. Cox, M.S. 2001. The Lancaster soil test method as an alternative to the Mehlich 3 soil test method. Soil Sci. 166:484–489.

10. Cushman, K., M. Maqbool., P. Gerard., E. Bedir., H. Lata, and R. Moraes. 2003. Variation of podophyllotoxin in leaves of Eastern Red Cedar (*J. virginiana)*. Planta Med. 69:1-2.

11. Dunford, N.T., S. Hiziroglu, and R. Holcomb. 2006. Effect of age on the distribution of oil in Eastern red cedar tree segments. Bioresource Technol. 98(14):2636-2640.

12. Eller, F. J., and J. W. King. 2000. Supercritical carbon dioxide extraction of cedarwood oil: a study of extraction parameters and oil characteristics. Phytochemical analysis 11:226-231.

13. Flake, R.H., E.V. Rudloff, and B.L. Turner. 2007. Quantitative study of clinical variation in *Juniperus virginiana* using terpenoid data. P. Natl. Acad. Sci. USA. 64:487-94.

14. Foster, S. 1993. Medicinal plant conservation and genetic resources: Examples from the temperate northern hemisphere. Acta. Hort. 330:67-73.

15. Gold, M.A., L.D. Godsey, and M. M. Cernusca. 2005. Competitive market analysis of eastern redcedar. Forest Prod. J. 55 (12):58-65.

16. Grinnell, G.B. 1962. The Cheyenne Indians. 2 Vols. Cooper Square Publishers, New York, New York.

17. Hart, J. A. 1976. Montana native plants and early peoples. Montana Historical Society, Helena, Montana.

18. Hellson, J.C. 1974. Ethnobotany of the Blackfoot Indians. National Museum of Man, Mercury Series, Canadian Ethnology Service Paper. 19.

19. Holm, Y., I. Laakso., R. Hiltunen, and B. Galambosi. 1997. Variation in the essential oil composition of *Artemisia annua* L. of different origin cultivated in Finland. Flavour Frag. J. 12:241-246.

20. Imbert, F. 1998. Discovery of podophyllotoxins. Biochimie 80:207-222.

21. Johnston, A. 1970. Blackfoot Indian utilization of the flora of the northwestern Great Plains. Economic Botany. 24:301-24.

22. Kindscher, K. 1992. Medicinal wild plants of the prairie. An ethnobotanical guide. University Press of Kansas. 340 pp.

23. Konuklugil, B. 1996a. Aryltetralin lignans from genus *Linum.* 1996. Fitoterapia. LXVII: 379-381.

24. Konuklugil, B. 1996b. Investigation of podophyllotoxin in some plants in Lamiaceae using HPLC. J. Fac. Pharm. Ankara. 25: 23-27.

25. Kroeber, A.L. 1908. The ethnology of the Gros Ventre. American Museum of Natural History, Anthropological Papers. 1:145-281.

26. Kuhnt, M., H. Rimpler, and M. Henrich. 1994. Lignans and other compounds from the mixed Indian medicinal plant *Hyptis verticillata.* Phytochemistry 36:485-489.

27. Kumar, J., and S. B. Parmar. 1996. Physicochemical and chemical variation in neem oils and some bioactivity leads against *Spodoptera litura* F. J Agric Food Chem. 44 (8):2137 - 2143.

28. Kupchan, S. M., J. C. Hemingway, and J. R. Knox. 1965. Tumor inhibitors VII. Podophyllotoxin, the active principle of *Juniperus virginiana.* J. Pharm. Sci. 54:659-660.

29. Lawler, I. R., W. J. Foley., B. M. Eschler., D. M. Pass., and K. Handasyde. 1998. Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. Oecologia. 116(1-2): 160-169.

30. Leander, K., and B. Rosen. 1988. Medicinal used for podophyllotoxin. US patent 4,788,216.

31. Lerndal, T., and B. Svensson. 2000. A clinical study of CPH 82 vs. methotrexate in early rheumatoid arthritis. Rheumatology (Oxford). 39: 316.

32. Liu, Z., S.B. Carpenter., W.J. Bourgeois., Y. Yu., R.J. Constantin., M.J. Falcon, and J.C. Adams. 1998. Variations in the secondary metabolite camptothecin in relation to tissue age and season in *Camptotheca acuminate*. Tree Physiol. 18(4):265-270.

33. McClintock, W. 1909. Materia medica of the Blackfeet. Zeitschrift fur Ethnologie. 273-279.

34. McGregor, R.L., T.M. Barkley., R.E. Brooks., and E.K. Schofield. 1985. Flora of the Great Plains. Great Plains Flora Association. University Press of Kansas. 1402 pp.

35. Meloni, M., D. Perini., R. Filigheddu, and G. Binelli. 2006. Genetic variation in five mediterranean populations of *Juniperus phoenicea* as revealed by inter-simple sequence repeat (ISSR) Markers. Ann. Bot. 97:299–304.

36. Moraes, R. M., C. Burandt., M. Ganzera., S. Li., I. Khan., and C. Canel. 2001. The American Mayapple revisited-Podophyllum peltatum-still a potential cash crop? Econ. Bot. 54:471-476.

37. Muranaka, T. M., K. Miyata, and Tachibana S. 1998. Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and biogenetic precursor. Phytochemistry 49: 491-496.

38. Muzquiz, M., C. Cuadrado., G. Ayet., C. Cuadra., C. Burbano, and A. Osagiel. 1994. Variation of alkaloid components of lupin seeds in 49 genotypes of *Lupinus albus* L. from different countries and locations. J. Agri. Food Chem. 42:1447-1450.

39. Payne, K. W., R. Wittwear., S. Anderson., E. J. Eisenbraun. 1999. Use of a modified abderhalden apparatus for comparing laboratory and industrial methods for isolating eastern redcedar essential oil. Forest Products Journal. 49: 90-92.

40. Pugh, N., I. Khan., R. M. Moraes, and D. Pasco. 2001. Podophyllotoxin lignans enhance IL-1 but suppress TNF-a mRNA expression in LPS-treated monocytes. Immuno-pharmacol. Immuno-toxicol. 23:83-95.

41. Purohit, M.C., R. Bahuguna., U.C. Maithani., A.N. Purohit, and M.S.M. Rawat. 1999. Variation in podophylloresin and podophyllotoxin contents in different populations of *Podophyllum hexandrum.* Curr. Sci. 77(8):1078.

42. Rai, L.K., P. Prasad, and E. Sharma. 2000. Conservation threats to some medicinal plants of the Sikkim Himalaya. Biol. Conserv. 93:27-33.

43. Roger, L., S. Fuller., T. Treloar., L. Stevenson., D. Hunter., T. Nevenimo., J. Binifa, and M. John. 2008. Characterization of tree-to-tree variation in morphological, nutritional and medicinal properties of *Canarium indicum* nuts. Agroforest. Syst. 73: 77–87.

44. San, Feliciano, A. J., M. M. Del Corral., M. Gordaliza, and M. A. Castro. 1989a. Acetylated lignans from *Juniperus sabinai.* Phytochemistry 28: 659-660.

45. San Feliciano, M. Medarde., J. L. Lopez., P. Puebla., J. M. M. Del Corral, and A. F. Barrero. 1989b. Lignans from *Juniperus thujifera*. Phytochemistry 28: 2863-2866.

46. Semen, H. and S. Hiziroglu. 2005. Production, yield and derivatives of volatile oils from Eastern red cedar (*Juniperus virginiana* L.). Am. J. Environ. Sci. 1(2):133-138.

47. Setzer, W.N., K.W. Whitaker, and R.O. Lawton. 1992. A chemical ecological study of the components of the essential oil of Eastern red cedar (*Juniperus virginiana*) from three habitats in Huntsville, Alabama. Castanea. 57(3):209-213.

48. Stahelin, H. F. and A. V. Wartburg. 1991. The chemical and biological route from podophyllotoxin to etoposide. Cancer Res. 51: 5-15.

49. Stephens, H.A. 1973. Woody plants of the north central plains. The University Press of Kansas. 530 pp.

50. Stewart, R.A. 2003. Physiographic regions of Mississippi. Handout, Department of Biological Sciences, Delta State University, 6 pp.

51. Von Rudloff E. 1975. Volatile leaf oil analysis in chemosynthetic studies of North American conifer. Biochem. System. Ecol**.** 2: 131-167.

52. Wallaart, T.E., N. Pras., A.C. Beekman, and W.J. Quax. 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Med. 66(1):57-62.

53. Wheeler, G.S., K.M. Ordung., R.M. Giblin-Davis, and P.D. Pratt. 2007. Intraspecific variation of *Melaleuca quinquenervia* leaf oils in its naturalized range in Florida, the Caribbean, and Hawaii. Biochem. Syst. Ecol. 35(8):489-500.

54. Williamson, R.E. and G.R. Gwynn. 1982. Variation of polyphenols in flue-cured tobacco cultivars attributed to location, stalk position, and year. Crop Sci. 22:144-146.

55. Yu P L Wang and Z. Chen 1991. A new podophyllotoxin-type lignan from *Dysosma versipellis* var. *Tomentosa.* J. Nat. Prod. 54:1422-1424.